Exhibit 18

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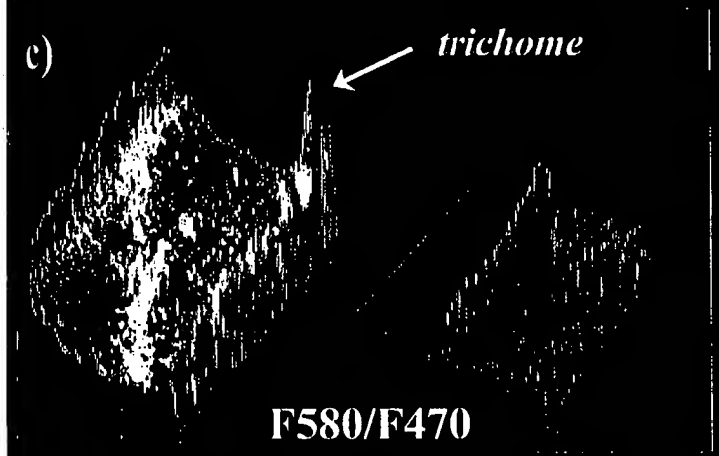

Flavonoid distribution in leaves. (see p. 366)

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Topical Application of 5-Aminolevulinic Acid and its Methylester, Hexylester and Octylester Derivatives: Considerations for Dosimetry in Mouse Skin Model¶

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ABSTRACT

Ester derivatives of 5-aminolevulinic acid (ALA-esters) have been proposed as alternative drugs for ALA in photodynamic therapy. After topical application of creams containing ALA, ALA methylester (ALA-Me), ALA hexylester (ALA-Hex) and ALA octylester (ALA-Oct) on mouse skin, typical fluorescence excitation and emission spectra of protoporphyrin IX (PpIX) were recorded, exhibiting a similar spectral shape for all the drugs in the range of concentrations (0.5–20%) studied. The accumulation kinetics of PpIX followed nearly a similar profile for all the drug formulations. The fluorescence of PpIX peaked at around 6–12 h of continuous cream application. Nevertheless, some differences in pharmacokinetics were noticed. For ALA cream, the highest PpIX fluorescence was achieved using 20% of ALA in an ointment. Conversely, 10% of ALA-Me and ALA-Hex, but not of ALA-Oct, in the cream was more efficient ($P < 0.05$) than was 20%. The cream becomes rather fluid when 20% of any of these ALA-esters is used in ointment, whereas 10% and lower concentrations of ALA-esters do not significantly increase fluidity of the cream. The dependence of PpIX accumulation on the concentration of ALA and ALA-ester in the applied cream followed ($P < 0.002$) kinetics as described by a mathematical model based on the Michaelis–Menten equation for enzymatic processes. Under the present conditions, the PpIX amount in the skin increased by around 50% by the application of ALA-Me, ALA-Hex or ALA-Oct for 4–12 h as compared with ALA for the same period. Observations of the mice under exposure to blue light showed that after 8–24 h of continuous application of ALA, the whole mouse was fluorescent, whereas in the case of ALA-Me, ALA-Hex and ALA-Oct the fluorescence of PpIX was located only at the area of initial cream application. The amount of the active compound in the applied cream necessary to induce 90% of the maximal amount of PpIX was determined for normal mouse skin. Optimal PpIX fluorescence can be attained using around 5% ALA, 10% ALA-Me and 5% ALA-Hex creams during short application times (2–4 h). Topical application of ALA-Oct may not gain optimal PpIX accumulation for short applications (<5 h). For long application times (8–12 h), it seems that around 1% ALA, 4% ALA-Me, 6% ALA-Hex and 16% ALA-Oct can give optimal PpIX fluorescence. But for long application times and high concentrations, systemic effect of ALA applied topically on relatively large areas should be considered.

INTRODUCTION

Photodynamic therapy (PDT) of tumors combines the administration of a photosensitizer, usually a porphyrin, and light exposure (1,2). A major drawback of PDT is the long-lasting skin sensitivity to sunlight for up to 2–4 weeks after treatment, which was reported for patients treated with hematoporphyrin derivatives and photofrin (3,4). A major drawback is less for the endogenous photosensitizer protoporphyrin IX (PpIX). Free PpIX has been found to clear faster from the body, exhibiting half-life of around 12 h after systemic administration (5,6). Accumulation of the endogenous sensitizer PpIX in tissues may be attained by the exogenous administration of its natural precursor 5-aminolevulinic acid (ALA). Normally, in the heme biosynthesis cycle, endogenous levels of ALA and PpIX are tightly regulated. ALA administered exogenously bypasses this feedback control, and consequently, free PpIX accumulates in the cells (7,8). The so-called ALA-PDT has been introduced in clinical practice (9,10).

PDT with topical application of ALA has been shown to give good cure rates for various superficial skin disorders (11–14). But topical application shows low cure rates for nodular tumors (10,15). The distribution of topically applied ALA in skin is dependent on many parameters, such as drug permeability through the stratum corneum, diffusion through dermis and epidermis, drug clearance time and conversion rate of ALA into PpIX (16). The limited penetration depth

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Abbreviations: ALA, 5-aminolevulinic acid; ALA-Hex, ALA hexylester; ALA-Me, ALA methylester; ALA-Oct, ALA octylester; PDT, photodynamic therapy; PpIX, protoporphyrin IX.

of ALA molecules in skin is a major drawback of topical application (17,18). ALA, being a hydrophilic molecule, is expected to penetrate into tissues in the depth range of 3 mm after 3–15 h of topical application (19). Ester derivatives of ALA (ALA-esters) have been found to induce PpIX more efficiently in cells *in vitro* than ALA (20,21). The idea behind the introduction of ALA-esters was that the esters should penetrate deeper into tissues because they are more lipophilic (22). ALA methylester (ALA-Me), ALA hexylester (ALA-Hex) and longer-chain esters of ALA are currently being studied by different investigators (23–26). But differences in biodistribution and pharmacokinetics of ALA and ALA-esters are still not well understood and are of great interest to be further explored. In the present work we have studied the pharmacokinetics of PpIX induced after topical application of ALA and some of its ester derivatives under different conditions in an *in vivo* model using normal skin of hairless mice. Topical application of ALA was used because this method of administration is common in the treatment of skin tumors and is advantageous over systemic administration because overall body sensitization is avoided and also high systemic concentrations of ALA may be slightly neurotoxic (27,28). The amount of PpIX formed in the skin was determined by means of a fluorescence spectroscopy, which is an efficient method for noninvasive tissue imaging *in vivo* (29,30). Considerations for dosimetry parameters such as the drug concentration and the application time are discussed on the basis of the experimental results presented.

MATERIALS AND METHODS

Chemicals. ALA and ALA-Me were purchased from Sigma Chemical Co. (St. Louis, MO). ALA-Hex and ALA octylester (ALA-Oct) were kindly supplied by PhotoCure ASA (Oslo, Norway).

Animals. Female Balb/c athymic nude mice were obtained from the animal department of the Norwegian Radium Hospital (Oslo, Norway). At the start of the experiments the mice were 7–8 weeks old, with average body weight of 25 g. Three mice were housed per cage with autoclaved filter covers in a room with subdued light at constant temperature (24–26°C) and humidity (30–50%). Food and bedding were sterilized, and the mice were given food and water *ad libitum.*

Continuous application of ALA, ALA-Me, ALA-Hex and ALA-Oct. For topical application, creams were prepared using 0.5%, 2%, 5%, 10% and 20% (wt/wt) of ALA, ALA-Me, ALA-Hex and ALA-Oct in a standard ointment (Unguentum, Merck, Darmstad, Germany). To facilitate proper application of the creams, the animals were anesthetized with subcutaneous injection of solution of Hypnorm (Janssen Pharmaceutica B.V., Tilburg, The Netherlands) and Dormicum (Hoffmann-La Roche AG, Basel, Switzerland) (1:1 vol/vol) with a lowest possible single bolus (0.02–0.03 mL per mouse). The animals woke up within 20 min and appeared normally active during the rest of the application time of ALA and its derivatives. Approximately, 25 ± 3 mg/cm² of the freshly prepared cream was continuously applied topically on a spot of approximately 1.5 cm diameter, which remained constant within the period of application (0–24 h) on the right flank of each mouse, and covered with transparent adhesive dressing (OpSite Flexifix, Smith & Nephew Medical Ltd., Hull, UK). The left flank of the mice was used as a control for systemic action of the drugs.

Fluorescence measurements. Fluorescence *in vivo* was measured noninvasively with a fiber-optic probe coupled to a luminescence spectrometer (LS50B, Perkin–Elmer, Norwalk, CT) equipped with a photomultiplier tube R3896 (Hamamatsu, Japan). The fiber-optic probe is based on a commercially available fiber accessory (Perkin–Elmer; two 1 m fused-silica fiber bundles joined in parallel at the measuring tip) fitted with a cylinder-shaped aluminum spacer (6.5 mm diameter), which provides a constant fixed distance of 10 mm between the fibers and the sample. This assures a relatively uniform light distribution over the measuring area and provides the maximum fluorescence signal for the given setup. Fluorescence intensity was measured at the cream application site as a function of time. Excitation wavelength was set at 407 nm, corresponding to the maximum of the Soret band of PpIX excitation spectrum in mouse skin, and fluorescence emission was measured at 635 nm. In addition, fluorescence excitation and emission spectra were measured to verify that the fluorescence signal originated mainly from PpIX. The 407 nm excitation light from the luminescence spectrometer was of low intensity (less than 1 mW/cm²) and did not induce any significant photobleaching of PpIX. Excitation and emission slits were set at 5 and 10 nm, respectively. Scattered excitation light was blocked from detection light with 515 nm cut off filter built in the luminescence spectrometer. Fluorescence measurements were carried out through the transparent occlusive dressing, which did not distort the fluorescence signal. Before cream application, fluorescence background (autofluorescence) of skin was recorded from each animal and was subtracted from the fluorescence data.

The animals were inspected for systemic action of the drugs under exposure to blue light (350–400 nm with maximum at 370 nm, around 5 mW/cm², TLD 18W/08; Philips, Holland) for a short period of time, which did not cause any significant photobleaching, in the darkness.

Data analysis. The data are averages from three mice for each group (three readings for each mouse). Accumulation of PpIX was visualized by plotting fluorescence intensity as a function of application time and drug concentration. The latter curves were fitted with a mathematical dose-dependent model using SigmaPlot 4.0 software (SPSS Inc., Chicago, IL):

$$F = \frac{F_{max} \cdot C}{C_M + C}, \qquad (1)$$

where F is the measured fluorescence intensity, C is the concentration (%) of the drug in the applied cream, F_{max} is the maximal intensity of PpIX fluorescence that can be achieved after cream application and C_M is the constant showing the concentration ($C = C_M$) of the active drug in the applied cream that induces 50% of the maximal amount of PpIX ($F = \frac{1}{2}F_{max}$).

Significance in differences between the data points was tested using the Student's t-test.

RESULTS

PpIX accumulation in mouse skin after the treatment with ALA and its ester derivatives

Typical fluorescence emission and excitation spectra of PpIX were observed in mouse skin after topical application of the creams containing ALA or ALA-esters. The spectral shape of the fluorescence was the same within the resolution of the instrumental setup for all the concentrations and the drugs studied (Fig. 1), showing that the fluorescence predominately originates from PpIX, which is the main final product under normal conditions.

Fluorescence of PpIX induced by ALA, ALA-Me, ALA-Hex and ALA-Oct peaked at around 6–12 h after cream application (Fig. 2). Within the limits of error accumulation, the kinetics of PpIX followed a nearly similar profile for all ALA concentrations studied (Fig. 2). There was a slight tendency for a lag phase after the application of ALA-Me and ALA-Oct. For all the ALA-esters the lowest concentrations (0.5–2%) gave significantly ($P < 0.05$) lower fluorescence than did the higher concentrations (Fig. 2).

Observation under exposure to the blue light (Fig. 3) showed that the whole mouse was fluorescent in the case of topical application (8 h) of ALA, whereas only the area of

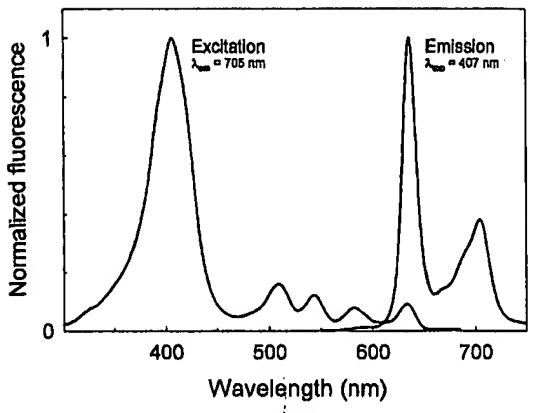

Figure 1. Fluorescence excitation and emission spectra of PpIX recorded in normal mouse skin after topical application of cream containing ALA or ALA-esters. Fluorescence emission spectrum is corrected for the spectral sensitivity of the detection system. Only one excitation and one emission spectrum are given because the spectra completely overlap after the application of ALA and its ester derivatives.

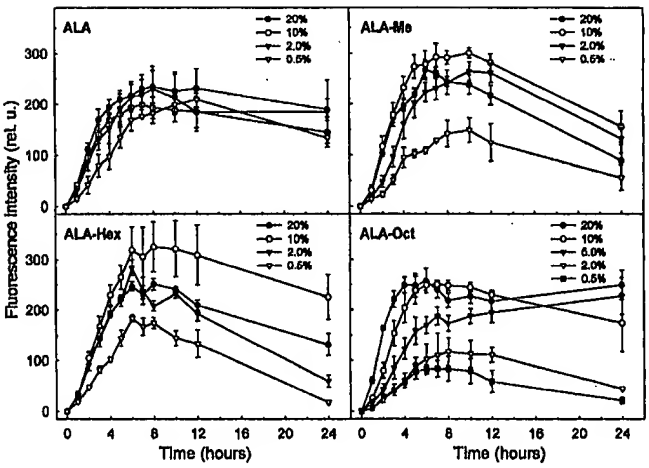

Figure 2. Kinetics of PpIX accumulation in normal mouse skin after topical application of creams containing various concentrations of ALA and ALA-esters. Error bars represent ±SE.

the initial cream application exhibited red fluorescence in the case of ALA-Me, ALA-Hex and ALA-Oct.

Influence of the drug concentration in the applied cream on PpIX accumulation

The accumulation of PpIX in the skin as a function of the concentration of the drugs was plotted, and the data were fitted ($P < 0.002$) using Eq. 1 (Fig. 4). The discrepancy between the 20% point for ALA-Me, ALA-Hex and ALA-Oct and theoretical fits (Fig. 4) can be explained by the fact that the creams become rather fluid when 20% of any of these ALA-esters are used in the cream. The data for ALA-Oct (0.5–5%, Fig. 4) were significantly ($P < 0.05$) different from that of the other drugs.

Concentrations for optimal PpIX fluorescence were determined from Fig. 4. The parameters F_{max} and C_M were calculated for each compound (Fig. 5) and, for practical reasons, the amount of the drug in the applied cream necessary to induce 90% of the maximal fluorescence of PpIX was estimated (Fig. 5, lower panel, right ordinate). The curve for ALA-Oct was significantly different from the other ones (Fig. 5, lower panel).

DISCUSSION

Recent studies showed that the sensitization of skin tumors with endogenous porphyrins can be made more selective by using lipophilic ester derivatives of ALA rather than ALA (24,25,31). But using comparable doses of ALA and its ester derivatives in animal models *in vivo,* it was found that ALA-esters do not induce significantly different amounts of PpIX compared with ALA (32,33). Nevertheless, some differences in pharmacokinetics do exist. Notably, the highest PpIX fluorescence was achieved using 20% of ALA in the ointment, whereas for ALA-Me and ALA-Hex the highest fluorescence was achieved with 10% drug (Fig. 2). This may partly be explained by the fact that the creams containing high concentrations (20%) of ALA-esters are slightly more fluid than ALA-cream. Application of a cream with 0.5% ALA gave almost as much fluorescence as application of a cream with 20% ALA, whereas for all esters the lowest concentration (0.5%) gave much lower fluorescence (Fig. 2). These differences may be related to the fact that the esters are more lipophilic than ALA (22), and a significant fraction of the ALA-esters may be bound in the stratum corneum and other lipophilic cellular compartments, thus slowing down

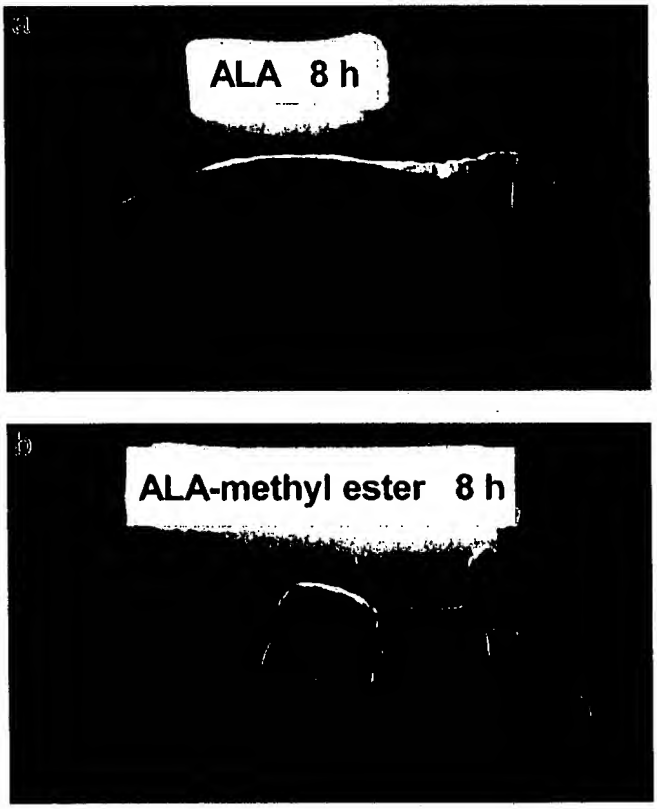

Figure 3. Difference in action of the topically applied drugs—a: Systemic action of ALA. b: ALA-Me induces PpIX fluorescence only in the area where the cream had been applied. The photos for ALA-Hex and ALA-Oct are not shown because their action was similar to that of ALA-Me. The area of the cream application is marked on the mice. Photographed in the darkroom under blue-light exposure.

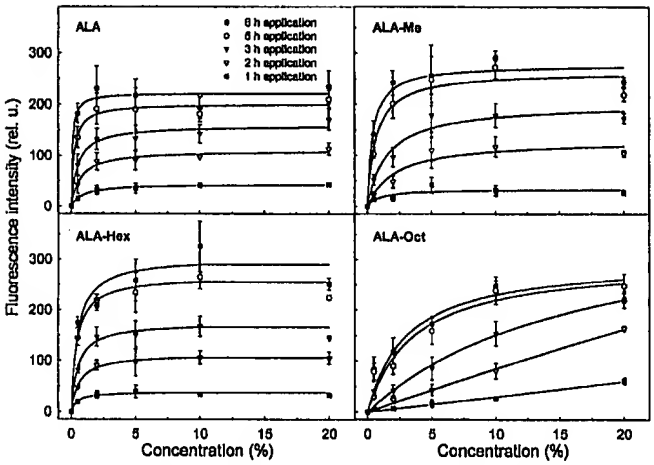

Figure 4. Dependence of PpIX accumulation on concentrations of ALA and ALA-esters in the cream applied topically on normal mouse skin. Error bars represent ±SE.

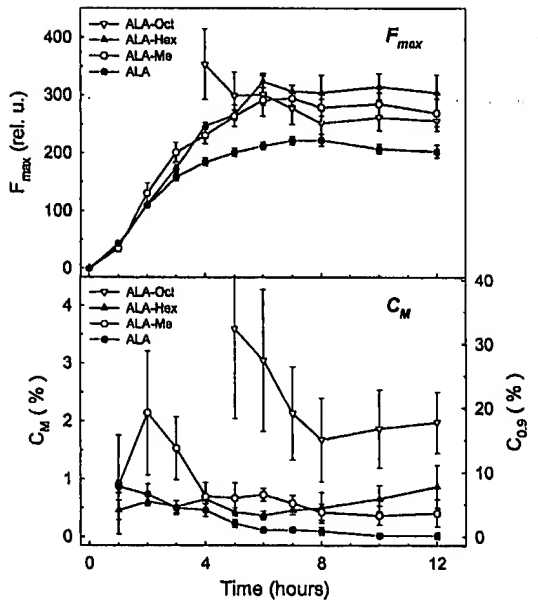

Figure 5. Time dependence of the parameters calculated by Eq. 1. F_{max} (upper panel)—the highest PpIX fluorescence possible to attain during continuous topical application of the cream containing ALA or ALA-esters. C_M (lower panel)—concentration of the active compound (ALA or ALA-esters) in the cream sufficient to induce 50% (left ordinate) and 90% (right ordinate) of the maximal PpIX amount corresponding to a certain time point. The constants F_{max} and C_M retained irrelevant values for ALA-Oct for $t < 5$ h, meaning that ALA-Oct could not gain optimal PpIX fluorescence during short application times. Error bars represent ±SE.

their penetration and production of PpIX. This is in agreement with our earlier finding that ALA-Me produced PpIX with a time lag as compared with ALA (32). The same tendency was seen in the present work, although the difference was smaller (Fig. 2). The reason could be the difference in application of anesthesia used in our present and previous work (32). Anesthesia of mice is necessary to facilitate application of creams. But recently, we found that systemic anesthesia of mice results in a decrease in the skin temperature and that the temperature is an important factor for PpIX synthesis after the application of ALA and ALA-esters (34). In the present study we used the lowest possible doses of anesthetics, *i.e.* significantly lower than in our previous work (32). Moreover, the discrepancy between these findings leads to a conclusion that the bioavailability of ALA-Me, and probably also of the other ALA-ester derivatives, is affected more by external and internal factors, such as temperature and intactness of the stratum corneum, than that of ALA. The role of the stratum corneum as a barrier for topically applied drugs has been demonstrated. The use of a penetration enhancer or tape-stripping of the stratum corneum of mice enhanced the production of PpIX more for ALA-Hex than for ALA (31). This indicates that ALA-Hex diffuses more slowly across the stratum corneum than does ALA. It should be noted that tape-stripping revealed that in all cases PpIX is found in the epidermis and not in the stratum corneum (31).

Kinetics on the long time scale (12–24 h of topical application) show lower PpIX fluorescence for low concentrations of ALA-esters than for ALA, where PpIX levels are almost as high at 24 h of application as at 12 h of application for all concentrations of ALA (Fig. 2). Furthermore, high concentrations of ALA-Hex and ALA-Oct, but not of ALA-Me, induced slightly higher levels of PpIX than did ALA after 24 h of application (Fig. 2). The clearance rate of PpIX is faster after the application of ALA-Me than after the application of ALA (35). ALA ester derivatives produce PpIX only on the spot of cream application, whereas ALA acts systemically (Fig. 3), *i.e.* after prolonged application (>5 h) it goes into circulation and produces PpIX in remote untreated skin areas in hairless mice (32,36). Possibly, for long application times the systemic action of topically applied ALA may contribute to PpIX levels also in the treated spot on mouse skin, which might explain the slower clearance of PpIX after the application of ALA than after the application of ALA-esters.

Aalders *et al.* (37) recently reported that experimentally determined fluorescence kinetics could be accurately described with the dose-dependent Michaelis–Menten model. For short application times (1–8 h) the dependence of PpIX fluorescence on the concentrations of ALA and ALA-esters in the applied cream is well described by the equation of conventional enzyme kinetics (Eq. 1) (Fig. 4). Parameters characterizing the conversion of ALA and ALA-esters into PpIX in our experiment are calculated using this approach (Fig. 5). Unfortunately, only a marginal knowledge is available concerning the pharmacokinetics of exogenously administered ALA and the relationship between the pharmacokinetics of ALA and ALA-induced PpIX in tissues *in vivo* (10). On the basis of the currently available data, one can speculate that for topical application of ALA and its ester derivatives, production of PpIX is a dominating process for application times of 1–8 h, whereas for longer times (>12 h) clearance is a dominant process (Fig. 2) (32,35).

In conclusion, under the present conditions and in the present animal model *in vivo* (normal mouse skin), the amount of PpIX in the skin increases around 1.5-fold during application from 4 to 12 h for ALA-Me, ALA-Hex or ALA-Oct as compared with ALA (Fig. 5). Practically 90% of the maximal amount of PpIX is achieved at concentrations lower than those commonly used in the clinics (Fig. 5). Creams

containing around 5% ALA, 10% ALA-Me and 5% ALA-Hex give optimal PpIX fluorescence during short application times (2–4 h). Topical application of ALA-Oct for short times (<5 h) does not give optimal PpIX fluorescence (Fig. 5). It seems that for long application times (8–12 h), creams containing around 1% ALA, 4% ALA-Me, 6% ALA-Hex and 16% ALA-Oct give optimal PpIX fluorescence. For long application times and for high concentrations, the systemic effect of topically applied ALA should be considered. But a simple Michaelis–Menten fit indicates the saturation of the enzymatic process, and increased availability of ALA may not increase the amount of PpIX over the basic capacity of the cells to synthesize porphyrins from ALA.

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